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(54) Title: DIAGNOSIS OF PRION DISEASES

(57) Abstract

Prion diseases, in which abnormal prions are implicated, are detected or measured by taking a blood sample from an animal or human and separating therefrom mononuclear cells. The abnormal prions are detected in the cell lysate or after isolating prion protein therefrom by any known technique, especially immunologically using antibodies to abnormal prion protein and enhanced chemiluminescent assay or immuno–PCR.

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DIAGNOSIS OF PRION DISEASES

1. Field of the Invention

This invention relates to the diagnosis of prion diseases, being those in which prions are implicated, especially neurodegenerative diseases and more especially bovine spongiform encephalopathy (BSE).

2. Description of the Related Art

It is now well accepted that certain diseases are caused by "proteinaceous infectious particles", termed "prions". For a review, see S.B. Prusiner, Scientific American, 30-37 (January 1995), the disclosure of which in relation to prion diseases is herein incorporated by reference. These diseases include principally neurodegenerative diseases, especially encephalopathies such as BSE in cattle, scrapie in sheep and goats and Creutzfeld-Jakob Disease (CJD) and CJD variants in humans. Prion proteins are present in the host cells of healthy individuals and are called PrP^{C}. In diseased individuals, an abnormal isoform of the protein (PrP^{Sc}) accumulates in the brain. The abnormal isoform is distinguished from the cellular form by its insolubility and partial resistance to proteases. It appears to be a conformational isomer. No satisfactory test has yet been devised for the early diagnosis of prion diseases in tissue samples from live animals (or humans).

SUMMARY OF THE INVENTION

According to the invention, the above problem is solved by a method which comprises assaying abnormal prion proteins in a sample of mononuclear cells present in the blood. The term "assay" is used herein to cover mere detection (the likely ordinary use envisaged) and a quantative or semi-quantative procedure in which some estimate is made of the amount of the abnormal prion protein present in the sample. Ordinarily the assay will be immunological, i.e. will depend on an interac-

tion between abnormal prion protein and an antibody thereto. The invention depends for its success on the application of the assay to samples concentrated in the specialised mononuclear cells, which will usually be monocytes.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The method of the invention may be applied to a concentrated sample of mononuclear cells separated from other blood cells. The mononuclear cells include particularly monocytes and macrophages. Monocytes, which originate in the bone marrow, are present in about 6 percent of white blood cells. Their residence time in blood is only 1 or 2 days, after which, as monocytes or macrophages, they squeeze through the cells lining the capillaries of the blood system and migrate into the body tissues.

To practise the present invention, it is necessary to separate white blood cells from red blood cells and the mononuclear cells from polynuclear cells and other white blood cells. The mononuclear cells are lysed, e.g. with buffered detergent. The purpose of such separations is to concentrate the mononuclear cells so that the sample contains a detectable amount of abnormal prion protein. Thereafter, it may be desirable to separate the protein components of the sample and to separate the proteins, e.g. by gel electrophoresis or gel filtration chromatography.

The prion proteins present in the mononuclear cell sample (or protein components thereof) are most conveniently detected immunologically. That is, they are reacted in some way with an antibody which is labelled, directly or indirectly.

The term "antibody" as used herein includes polyclonal antiserum, monoclonal antibodies, fragments of antibodies such as Fab and genetically engineered anti-

bodies. The antibodies may be chimeric or of a single species. Antibodies raised against abnormal prion proteins or appropriate short peptides therefrom can be used. They include principally antibodies which react, e.g. by Western blotting or immunohistochemical detection with brain tissue taken from dead animals or humans which or who have died from the prion disease to be diagnosed by the method of the invention or another prion disease where the antibody cross-reacts with the prion protein of that other disease. Thus, BSE in cattle, scrapie in sheep and CJD (including new CJD variants) in humans may be detected, normally with antibodies raised against the prion protein from the brain tissue of the animal or human suffering from the same disease. Preferably, the brain tissue is first digested with a protease, such as proteinase K, to remove normal prion protein, before the tissue is used to raise antibodies.

Antibodies raised against brain tissue are not necessarily specific to abnormal prion protein. Specificity can be imparted to a polyclonal antiserum by back-absorbing it with a normal prion protein or a peptide thereof, whereby the unbound residue is enriched in epitopes specific to the abnormal prion protein. The enriched material is then tested for binding to brain tissue and to recombinantly-derived normal prion protein (see below) and is selected if it combines with both.

More conveniently, the antibody to the abnormal prion protein is prepared as described by C Korth *et al.*, Nature *390*, 74-77 (1997), the disclosure of which in relation to preparation and characterisation of antibodies is herein incorporated by reference. Mouse hybridomas are made by immunising mice with purified recombinant bovine or CJD prion protein. After fusion of mouse spleen cells with mouse myoloma cells by the

well-known Köhler-Milstein method, hybridomas are obtained. Supernatants from the hybridomas are screened with the recombinant bovine prion protein and the protease digested brain homogenate of BSE-diseased cattle. Using this methodology, Korth *et al.* produced an antibody "15B3" which they characterised as binding to three peptides having amino acid sequences from three distinct regions of bovine prion protein, namely at positions 142-148, 162-170 and 214-226. It is believed that these regions form a discontinuous epitope. A similar antibody to 15B3 can be produced by screening the hybridoma supernatants with these three peptides, bound to a support, selecting those hybridomas which bind to all three. The peptides differ slightly as between human and bovine prion proteins, so it would be best to select the peptide appropriate to the source of the recombinant prion protein. The supernatant from the hybridomas is then used as the source of monoclonal antibodies, in the conventional way.

The precise type of the immunological reaction is not critical, but preferably the prion protein is absorbed on a solid phase. Thus, it is conveniently transferred to a membrane and Western-blotted with the antibody. Alternatively, an immunoassay such as an ELISA may be carried out by immobilising the sample on a solid phase.

Western blotting (immunoblotting) may be carried out in any known way. The protein has to be isolated or at least semi-separated, e.g. by one dimensional gel electrophoresis. The protein is transferred to a suitable membrane, preferably of nitrocellulose or polyvinylidene difluoride, the membrane is blocked to prevent non-specific adsorptions, e.g. with milk powder. After blocking, the protein can be detected directly or indirectly. Direct detection uses labelled primary antibo-

dies (the anti-prion protein antibodies), but it is more convenient to use unlabelled primary antibodies and to label secondary antibodies which are anti- to the immunoglobulin of the primary antibodies. The antibodies are usually labelled with an enzyme such as peroxidase or alkaline phosphatase, either directly or *via* a ligand which has a high affinity for a co-ligand, such as biotin, which has a high affinity for streptavidin and avidin. The protein is then conveniently detected by adding an enzyme substrate, e.g. a colour-forming one, to read out the enzyme label or an enzyme-labelled co-ligand plus a substrate for the enzyme if a high affinity type ligand was used.

It may be desirable to digest the protein to be Western blotted with a protease such as proteinase K before it is put on the gel. The abnormal prion protein, being resistant to cleavage by proteinases will then have a higher molecular weight and travel less far on the gel than normal prion protein.

An alternative method of quantifying or detecting the presence of protein is the use of immunoassays, which may be performed on a lysate of the mononuclear cells, on protein components extracted therefrom or on isolated or semi-separated protein. Any known method of immunoassay may be used. An antibody capture assay is preferred. Here, the test sample is allowed to bind to a solid phase, such as a well of a plastics microtitre plate, and the anti-prion protein antibody is then added and allowed to bind. After washing away unbound material, the presence or amount of antibody bound to the solid phase is determined using a labelled second antibody, as described above. Alternatively, a competition assay could be performed between the sample and a labelled peptide having the same sequence as in a prion protein, these two antigens being in competition for a

limited amount of antibody bound to a solid support. The labelled peptide could be pre-incubated with the antibody on the solid phase, whereby the prion protein in the sample displaces part of the peptide bound to the antibody. Alternatively, the two antigens are allowed to compete in a single co-incubation with the antibody. After removal of unbound antigen from the support by washing, the amount of label attached to the support is determined and the abnormal prion protein in the sample is detected or measured by a reference to standard titration curves established previously.

The substrate for the enzyme may be colour-forming, fluorescent or chemiluminescent, for example.

It is highly preferable to use an amplified form of assay, whereby an enhanced "signal" is produced from a relatively low level of prion protein to be detected. One particular form of amplified immunoassay is enhanced chemiluminescent (ECL) assay. Here, the antibody is preferably labelled with horseradish peroxidase, which participates in a chemiluminescent reaction with luminol, a peroxide substrate and a compound which enhances the intensity and duration of the emitted light, typically 4-iodophenol or 4-hydroxycinnamic acid.

Another preferred form of amplified immunoassay is immuno-PCR. In this technique, the labelled antibody is covalently linked to a molecule of arbitrary DNA comprising PCR primers, whereby the DNA with the antibody attached to it is amplified by the polymerase chain reaction. See E. R. Hendrickson *et al.*, Nucleic Acids Research 23, 522-529 (1995) or T. Sano et al., in "Molecular Biology and Biotechnology" ed. Robert A. Meyers, VCH Publishers, Inc. (1995), pages 458-460, the disclosures of which in relation to immuno-PCR methods and reagents are herein incorporated by reference. The signal is read out as before.

The following Example illustrates the invention. "Millipore", "Seromed" and "Tween" are Registered Trade Marks.

EXAMPLE

In this Example, all operations are conducted under sterile conditions and an ambient temperature. A 20ml sample of blood is taken from an animal or human suspected to be suffering from a prion disease. The sample is taken using a syringe containing 1 percent of a 5000U/ml heparin preparation.

For each sample to be tested, two sterile 12ml tubes (Falcon 3033) are used. In each tube is placed 4ml of Ficoll-Paque (Pharmacia AB) and 8-9ml of the blood (to fill the tube to the upper mark) are squirted gently down the walls of the tubes. The stoppers of the tubes are flamed to sterilise them and the tubes are then closed. The tubes are centrifuged at 2500 rpm for 15 minutes. During this time, a Falcon tube is prepared which contains 2ml of TC199 Hanks' medium containing 0.35g/ml sodium bicarbonate, but no L-glutamine ("Seromed" F 0635 from Fakola AG), pre-filtered through a 0.22 micrometre "Millipore" filter). After centrifugation, the circular band of monocytes is recovered by use of a Pasteur pipette and placed in 2ml of TC199 Hanks' medium, ready for culture. The volume of the tube is made up to the upper mark with TC199 medium. The cells are washed three times with phosphate - buffered saline (PBS), pH 7.4 and centrifuged for 10 minutes each time at 1500, 1200 and 1000 rpm respectively. After each wash the cells are re-suspended with a Pasteur pipette. Finally, the cells are suspended in 2.5ml of RPMI 1640 medium containing 2.0g/l sodium bicarbonate, but without L-glutamine ("Seromed" F 1215 from Fakola AG), together with 20 percent AB serum.

To carry out the assay, one mg of mononuclear cell

protein samples is solubilized by mixing with 20µl of a solution containing SDS 2% (w/v), 5% (v/v) beta-mercaptoethanol, 10% (w/v) glycerol and Tris-HCI 62.5mM (pH 6.8). The mixture is heated at 95° C for 5 min. The proteins are then separated by 1-D SDS-PAGE and transferred onto PVDF membranes using a semi-dry apparatus with 10% methanol and 10mM CAPS as buffer (pH 11) at 50 V for 45 min. The membranes are stained with amido black, scanned and tested with the anti-abnormal prion protein antibody. The PVDF membranes are first blocked with a blocking solution (5% (w/v) dry low fat milk, 0.5% (v/v) "Tween 20" and 10mM PBS (pH 7.4)) for 30 min, probed in the same solution containing the primary antibody (anti-abnormal prion protein) for 2 h and washed for 3 X 10 min in 10mM PBS (pH 7.4) and 0.5% (v/v) Tween 20. The secondary goat anti-mouse or anti-rabbit antibody (1:100 BAKO) labelled with peroxidase is mixed with the blocking solution. The membranes are soaked in the latter solution for 1 h. and then washed for 4 X 10 min in 10mM PBS (pH 7.4) and 0.5% (v/v) "Tween 20". Protein detection is achieved using enhanced chemiluminescence and x-ray films as described by the manufacturer (Boehringer Mannheim). The membranes can be reprobed 3 to 5 times after stripping. Stripping of the membranes is achieved using a solution containing 2% (w/v) SDS, 200mM DTE and 63mM Tris-acetate (pH 6.7) at 50° C for 30 min and the membranes are washed for 4 X 10 min in 10mM PBS (pH 7.4). All the immunoblotting steps are done in glass cylinders (25cm X 6cm) with a hybridization oven at 25°C.

CLAIMS

1. A method of detecting or measuring abnormal prion proteins present in a sample of blood taken from an animal or human suspected of suffering from a prion disease, which comprises separating mononuclear cells present in the blood and detecting or measuring abnormal prion proteins present in said cells.

2. A method according to Claim 1, wherein the abnormal prion proteins are detected or measured immunologically.

3. A method according to Claim 2, wherein the prion proteins are detected or measured using an antibody to abnormal prion protein.

4. A method according to Claim 2 or 3, wherein the prion proteins are isolated or semi-separated from a lysate of the mononuclear cells and are then Western blotted.

5. A method according to any preceding claim for the detection of BSE from samples of blood taken from cattle.

6. A method according to any one of Claims 1 to 4 for the detection of CJD from samples of blood taken from humans.

7. A method according to any one of Claims 1 to 4 for the detection of scrapie from samples of blood taken from the sheep.

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/GB 97/03135

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/68

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 96 17250 A (MINI AGRICULTURE & FISHERIES ;DAWSON MICHAEL (GB); MARTIN TREVOR C) 6 June 1996 see the whole document	1
A	WO 93 23432 A (UNIV NEW YORK ;INST NAZIONALE NEUROLOGICO C B (IT)) 25 November 1993 see the whole document	1
P,A	WO 97 37227 A (INSTITUUT VOOR DIERHOUDERIJ EN DIERGEZONDHEID) 9 October 1997 see the whole document	1
P,A	WO 97 38315 A (US HEALTH ;HSICH GARY (US); KENNEY KIMBRA (US); GIBBS CLARENCE J () 16 October 1997 see the whole document	1

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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P,A	WO 97 10505 A (UNIV CALIFORNIA) 20 March 1997 see the whole document	1

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INTERNATIONAL SEARCH REPORT

..formation on patent family members

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